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ORIGINAL PAPER

Mutations in the Atp1p and Atp3p subunits of yeast ATP synthase differentially affect respiration and fermentation in *Saccharomyces cerevisiae*

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Abstract *ATP1-111*, a suppressor of the slow-growth phenotype of *yme1*Δ lacking mitochondrial DNA is due to the substitution of phenylalanine for valine at position 111 of the alpha-subunit of mitochondrial ATP synthase (Atp1p in yeast). The suppressing activity of *ATP1-111* requires intact beta (Atp2p) and gamma (Atp3p) subunits of mitochondrial ATP synthase, but not the stator stalk subunits b (Atp4p) and OSCP (Atp5p). *ATP1-111* and other similarly suppressing mutations in *ATP1* and *ATP3* increase the growth rate of wild-type strains lacking mitochondrial DNA. These suppressing mutations decrease the growth rate of yeast containing an intact mitochondrial chromosome on media requiring oxidative phosphorylation, but not when grown on fermentable media. Measurement of chronological aging of yeast in culture reveals that *ATP1* and *ATP3* suppressor alleles in strains that contain mitochondrial DNA are longer lived than the isogenic wild-type strain. In contrast, the chronological life span of yeast cells lacking mitochondrial DNA and containing these mutations is shorter than that of the isogenic wild-type strain. Spore viability of strains bearing *ATP1-111* is reduced compared to wild type, although *ATP1-111* enhances the survival of spores that lacked mitochondrial DNA.

Keywords ATP synthase · Yeast mitochondria · Spore viability · Chronological aging · Fermentation · Respiration

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Introduction

The budding yeast *Saccharomyces cerevisiae* is petite-positive as it is able to grow on a fermentable carbon source in the absence of mitochondrial DNA (mtDNA) (Faye et al., 1973). Strains with intact, fully-functional mtDNA (ρ^+ strains) can be converted into strains without mtDNA (ρ° strains) or with dysfunctional mtDNA (ρ^- strains) by inclusion of ethidium bromide (EtBr) in the growth media (Goldring et al., 1970; Nagley and Linnane, 1970). Both ρ° and ρ^- strains occur spontaneously in wild-type laboratory strains, and their appearance in culture can be increased by mutation of various nuclear genes (Chen and Clark-Walker, 2000). Other yeast species such as *Kluyveromyces lactis* and *Schizosaccharomyces pombe* that are unable to grow in the absence of mtDNA are petite-negative (Heslot et al., 1970; Clark-Walker and Chen, 1996). Because *S. cerevisiae* mtDNA encodes subunits of electron transport complexes and the F_o component of ATP synthase, no electron transport or oxidative phosphorylation is possible in ρ° or ρ^- strains. Deletion of particular nuclear genes from *S. cerevisiae* can also lead to defects in respiration or the destabilization of mtDNA (Heslot et al., 1970; Prescott et al., 1994; Clark-Walker and Chen, 1996); Giraud and Velours, 1997).

Yme1p is an AAA-protease in the inner mitochondrial membrane that has its ATPase and protease domains in the intermembrane space. It can act both as a chaperone and a protease, and its major role is in the formation, maintenance, and under some circumstances, degradation of protein complexes (Van Dyck and Langer, 1999; Langer et al., 2001). It also has a role in maintenance of the mtDNA in the matrix (Shafer et al., 1999). Yeast strains bearing *yme1*Δ grow extremely slowly when mtDNA is absent (Thorsness and

Fox, 1993; Thorsness et al., 1993). Mutations in the Rpt3p subunit of the proteasome and the Atp1p and Atp3p subunits of F_1-ATPase suppress the *yme1*$\Delta\rho^\circ$ slow-growth phenotype (Campbell et al., 1994; Weber et al., 1995; Kominsky and Thorsness, 2000). Mutations in F_1-ATPase subunits also allow *K. lactis* strains to become petite positive, and in the case of Atp3p they are the same mutations as those that suppress the *yme1*$\Delta\rho^\circ$ slow-growth phenotype in *S. cerevisiae* (Clark-Walker et al., 2000). An altered interaction between Atp3p and Atp1p has been proposed for the mutant strains of both *S. cerevisiae* and *K. lactis* (Clark-Walker et al., 2000; Kominsky and Thorsness, 2000). In ρ° cells F_1-ATPase has been postulated to act in consort with the adenine nucleotide translocator (ANT) in the maintenance of an inner mitochondrial membrane potential (Giraud and Velours, 1997; Buchet and Godinot, 1998). Mutations in Atp1p and Atp3p that increase *yme1*$\Delta\rho^\circ$ growth have an increased F_1-ATPase activity that presumably enhances the ANT-driven membrane potential (Kominsky et al., 2002). However, in another study no correlation between ATPase activity and suppressor activity was observed (Chen and Clark-Walker, 1999). Although these *ATP1* and *ATP3* mutations all act as suppressors of the slow-growth phenotype of *yme1*Δ strains that lack mtDNA, in other respects their properties vary. For example, *ATP3-5* is respiration incompetent whereas *ATP3-1* and *ATP1-75* are not (Weber et al., 1995).

Deletion of the F_1-ATPase inhibitor, Inh1p, partially suppresses the *yme1*$\Delta\rho^\circ$ slow-growth phenotype of *S. cerevisiae* (Kominsky et al., 2002). Since F_1-ATPase activity is required to establish a membrane potential in ρ° mitochondria (Giraud and Velours, 1997), and *yme1*$\Delta\rho^\circ$ strains have reduced F_1-ATPase activity (Kominsky et al., 2002), we hypothesized that the slow growth of the *yme1*$\Delta\rho^\circ$ strain resulted from an increase in the level of Inh1p and consequent loss of F_1-ATPase activity. Yme1p may regulate the level of Inh1p although the protease domain of Yme1p is located in the intermembrane space and Inh1p is in the matrix. To test this hypothesis, we measured the levels of myc-tagged Inh1p in strains with and without Yme1p, and isolated a suppressor of the *yme1*Δ *inh1*$\Delta\rho^\circ$ strain that allowed growth similar to wild type. Our results suggest that Yme1p does not exercise its influence on ρ° growth via Inh1p. The new Atp1p suppressor is mutated at a highly conserved position and supports the proposal that increased *yme1*$\Delta\rho^\circ$ growth is affected by alterations in the structure of F_1-ATPase, particularly through the interaction between Atp1p and Atp3p. We have examined the fitness of this and other suppressor mutations in terms of growth characteristics, chronological aging, and long-term spore viability to determine why these mutations have been strongly selected against during eukaryotic evolution.

Materials and methods

Strains, media, and growth of yeast

The genotypes of *S. cerevisiae* strains used in this study are listed in Table 1. Standard genetic techniques were used to construct and analyze the various strains (Sherman et al., 1986). *Escherichia coli* strain XL-1 Blue (Stratagene) was used for preparation and manipulation of DNA. Plasmid-containing *E. coli* were grown in Luria-Bertani (LB) broth supplemented with 125μg/ml ampicillin (Sambrook et al., 1989).

Yeast strains were grown in rich glucose medium (YPD) containing 2% glucose, 2% Bacto peptone, 1% yeast extract (Difco), 40 mg/l adenine and 40 mg/l tryptophan; rich ethanol glycerol medium (YPEG) containing 3% ethanol, 3% glycerol, 2% Bacto peptone, 1% yeast extract (Difco), 40 mg/l adenine and 40 mg/l tryptophan; rich raffinose medium (YPR) in which filter sterilized raffinose replaced glucose in the YPD formulation; synthetic glucose medium (SD) containing 2% glucose, 6.7 g/l Yeast Nitrogen Base without amino acids (Difco) supplemented with appropriate nutrients; synthetic ethanol glycerol medium (SEG) containing 3% ethanol, 3% glycerol, 6.7 g/l Yeast Nitrogen Base without amino acids (Difco) supplemented with appropriate nutrients; and sporulation medium (SPO) containing 1% potassium acetate supplemented with the complete set of nutrients. The complete set of nutrients is uracil 40 mg/l, adenine 40 mg/l, tryptophan 40 mg/l, lysine 60 mg/l, leucine 100 mg/l, histidine 20 mg/l, isoleucine 30 mg/l, and valine 150 mg/l. For plates, bacteriological agar (US Biological) was added at 15 g/l. Where indicated, ethidium bromide (EtBr) was added at 25 μg/ml and geneticin at 300 μg/ml, or nourseothricin (Werner Bioagents) was top spread on plates at 25 μg/ml. All yeast media were incubated at 30°C except SPO, which was incubated at room temperature. LB medium was incubated at 37°C. When ρ° strains were specifically used (Figs. 4B, 7, 8 and 9; Table 2), the corresponding ρ^+ strain was converted to ρ° by serial culturing in SD liquid media containing 25 μg/ml EtBr (Fox et al., 1991).

Suppressor mutations for *yme1*Δ *inh1*$\Delta\rho^\circ$ were isolated by taking individual colonies grown on YPEG plates and growing each in a 5 ml shaking SD + EtBr culture for 3 days. A 100 μl aliquot from each tube was put into 5 ml fresh SD + EtBr and shaken for another 3 days. From each tube in which growth was observed, 50 μl was put into 5 ml fresh SD + EtBr. After 7 days shaking, 50 μl was spread on SD + EtBr plates. After 5 days colonies were replica plated onto fresh SD + EtBr. Individual colonies from these plates were streaked for isolated colonies onto

Table 1 Yeast strains used in this study

Strain[a]	Genotype[b]	Source
BFY101	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 inh1Δ::kanMX6 [ρ+, TRP1]*	This study
BFY107	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 inh1Δ::kanMX6 [ρ+, TRP1]*	This study
BFY108	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 INH1-myc [ρ+, TRP1]*	This study
BFY109	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 INH1-myc [ρ+, TRP1]*	This study
BFY137	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 inh1Δ::kanMX6 ATP1-111[ρ+, TRP1]*	This study
BFY138	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-111[ρ+, TRP1]*	This study
BFY139	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-111[ρ+, TRP1]*	This study
BFY140	*MAT***a** *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-111[ρ+, TRP1]*	This study
BFY141	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP1-111[ρ+, TRP1]*	This study
BFY142	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 ATP1-111[ρ+, TRP1]*	This study
BFY146	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ::natMX4 atp1Δ::kanMX6 [ρ+, TRP1]*	This study
BFY148	*MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 ATP1-111 atp3Δ::kanMX6 [ρ+, TRP1]*	This study
BFY149	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP1-111 atp5Δ::kanMX6 [ρ+, TRP1]*	This study
BFY152	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ ATP1-111 atp4-Δ1::URA3[ρ+, TRP1]*	This study
BFY156	*MAT***a** *ura3-52 lys2 leu2-3,112 trp1-Δ yme1-Δ1::URA3[ρ+, TRP1]*	This study
BFY160	*MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-111 atp4-Δ1::URA3[ρ+, TRP1]*	This study
BFY165	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp4-Δ1::URA3 atp1Δ::kanMX6 [ρ+, TRP1]*	This study
BFY178	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP1-111 atp2Δ::kanMX6 [ρ+, TRP1]*	This study
BFY188	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-111 atp5Δ::kanMX6 [ρ+, TRP1]*	This study
BFY190	*MATα ura3-52 lys2 leu2–3,112 trp1-Δ1 yme1-Δ1::URA3 atp5Δ::kanMX6 [ρ+, TRP1]*	This study
DKY40	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ atp4-Δ1::URA3[ρ+, TRP1]*	(Kominsky et al., 2002)
DKY41	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ atp4-Δ1::URA3 [ρ+, TRP1]*	(Kominsky et al., 2002)
DKY48	*MATα ura3-52 ade2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 atp4-Δ1::URA3[ρ+, TRP1]*	(Kominsky et al., 2002)
KWY91	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP3-5[ρ+, TRP1]*	This study
KWY94	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP1–75[ρ+, TRP1]*	This study
KWY96	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP3–1[ρ+, TRP1]*	This study
KWY116	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ cat5Δ::kanMX6 ATP1-111 [ρ+, TRP1]*	This study
KWY117	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ cat5Δ::kanMX6 ATP1-75 [ρ+, TRP1]*	This study
KWY118	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ cat5Δ::kanMX6 ATP3-1 [ρ+, TRP1]*	This study
PTY33	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 [ρ+, TRP1]*	(Thorsness and Fox, 1993)
PTY44	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 [ρ+, TRP1]*	(Thorsness and Fox, 1993)
PTY52	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ+, TRP1]*	(Thorsness and Fox, 1993)
PTY60	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ+, TRP1]*	(Thorsness et al., 1993)
PTY93	*MAT***a** *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-75 [ρ+, TRP1]*	(Weber et al., 1995)
PTY100	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 ATP3-5 [ρ+, TRP1]*	(Weber et al., 1995)
PTY102	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-75 [ρ+, TRP1]*	This study
PTY109	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP3-1 [ρ+, TRP1]*	(Weber et al., 1995)
TCY1	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 cat5Δ::kanMX6 [ρ+, TRP1]*	This study
TCY37	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 atp3Δ::kanMX6 [ρ+, TRP1]*	This study
TCY48	*MAT***a** *ura3-52 ade2 leu2-3,112 trp1-Δ1 atp2Δ::kanMX6 [ρ+, TRP1]*	This study
TCY71	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 atp5Δ::kanMX6 [ρ+, TRP1]*	This study
TCY82	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::nat4MX4 [ρ+, TRP1]*	This study

[a] All strains are isogenic.
[b] The mitochondrial genotype is bracketed.

SD + EtBr. Suppressor candidates were crossed to *yme1Δ inh1Δ ρ+* and sporulated. Colonies from the resulting spores were assayed for growth on SD + EtBr after tetrads were picked.

The doubling times and yields of yeast strains were determined in shaker cultures by inoculating 50 ml of medium in 300 ml Nephelo Culture flasks (Wheaton) with 10^7 cells ($OD^{600} = 1 = 10^7$ cells/ml) from 5 ml stationary cultures in the same medium. OD was measured using a Klett Colorimeter (Scienceware) with a 640–700 nm filter. Use of Nephelo flasks allowed OD to be measured without removing samples, thus maintaining a constant sample volume. Doubling times were calculated from Klett readings measured during log phase of growth. Strains in all media were in log phase from Klett units 100–150 and this range was used for all readings. Yields were measured as OD^{600} after inoculation of 5 ml medium with 10^6 cells from the same medium and shaking either for 3 days (SD-complete)

Table 2 Doubling times during log phase (hours at 30°C)

Strain name	Relevant genotype	Media			
		YPD	SD-complete	YPEG	SEG-complete
PTY44	Wild Type	1.5 ± 0.1	2.6 ± 0.2	4.1 ± 0.1	4.7 ± 0.1
BFY141	*ATP1-111*	1.5 ± 0.1	2.6 ± 0.2	4.5 ± 0.2	5.7 ± 0.4
KWY94	*ATP1-75*	1.7 ± 0.1	3.0 ± 0.2	4.5 ± 0.1	5.2 ± 0.2
KWY96	*ATP3-1*	1.6 ± 0.1	2.8 ± 0.1	4.1 ± 0.1	5.0 ± 0.2
PTY100	*ATP3-5*	1.6 ± 0.1	2.9 ± 0.2	NG[a]	NG[a]
PTY52	*yme1*Δ	1.8 ± 0.1	3.0 ± 0.1	5.2 ± 0.2	5.7 ± 0.2
BFY138	*yme1*Δ *ATP1-111*	1.8 ± 0.1	3.0 ± 0.2	6.0 ± 0.3	6.7 ± 0.5
PTY93	*yme1*Δ *ATP1-75*	1.7 ± 0.1	3.1 ± 0.2	6.8 ± 0.3	8.2 ± 0.1
PTY109	*yme1*Δ *ATP3-1*	1.7 ± 0.1	3.1 ± 0.2	5.3 ± 0.3	6.8 ± 0.4
KWY91	*yme1*Δ *ATP3-5*	1.8 ± 0.2	3.3 ± 0.3	NG[a]	NG[a]
TCY1	*cat5*Δ	2.1 ± 0.1	2.8 ± 0.1		
KWY116	*cat5*Δ *ATP1-111*	2.0 ± 0.1	2.8 ± 0.1		
KWY117	*cat5*Δ *ATP1-75*		2.8 ± 0.1		
KWY118	*cat5*Δ *ATP3-1*		2.7 ± 0.2		
PTY44 (ρ°)	Wild Type ρ°	2.5 ± 0.1	3.5 ± 0.1		
BFY141 (ρ°)	*ATP1-111* ρ°	1.7 ± 0.1	3.0 ± 0.1		
KWY94 (ρ°)	*ATP1-75* ρ°	2.0 ± 0.1	2.7 ± 0.1		
KWY96 (ρ°)	*ATP3-1* ρ°	1.9 ± 0.1	2.8 ± 0.1		
PTY100 (ρ°)	*ATP3-5* ρ°	2.0 ± 0.1	2.8 ± 0.2		
BFY138 (ρ°)	*yme1*Δ *ATP1-111* ρ°	2.4 ± 0.1	3.2 ± 0.2		
PTY93 (ρ°)	*yme1*Δ *ATP1-75* ρ°	3.2 ± 0.2	3.8 ± 0.2		
PTY109 (ρ°)	*yme1*Δ *ATP3-1* ρ°	3.4 ± 0.2	4.2 ± 0.2		
KWY91 (ρ°)	*yme1*Δ *ATP3-5* ρ°	3.3 ± 0.2	3.6 ± 0.2		
TCY1 (ρ°)	*cat5*Δ ρ°	2.8 ± 0.1	3.6 ± 0.1		
KWY116 (ρ°)	*cat5*Δ *ATP1-111* ρ°	2.1 ± 0.1	2.7 ± 0.1		

[a] NG = Unable to grow in respiration media.

or 3, 6, and 8 days (SEG-complete) and by the number of colony forming units (CFU) at the same sampling times, obtained after dilution of a given volume of culture as described in the chronological aging assay.

Spore viability was assessed during prolonged incubation on SPO medium. Diploid yeast were first selected on SD plates, and then patched onto SPO plates. After 5, 15 and 40 days, tetrads were dissected onto YPD. Colonies grown from spores on YPD were counted and respiration competence was assessed by growth on YPEG plates.

The genetic state of mtDNA of yeast strains (ρ^+ or ρ^-/ρ°) was determined using one of two approaches. Strains bearing the *ade2* allele were plated at 300 colonies/plate on YPD not supplemented with adenine. Respiration competent colonies turned red while respiration incompetent colonies remained white. Strains bearing the wild-type *ADE2* allele were plated on YPD at the same density. After incubation colonies were replica plated to YPEG and incubated to determine the percentage of respiratory competent cells. In order to determine whether non-respiring colonies were ρ^+ or ρ^-/ρ°, they were crossed with a wild-type ρ° strain and the resulting diploids were assessed for their ability to grow on YPEG. Diploids unable to grow on YPEG indicated that the non-respiring haploid contained either a ρ^- or ρ° genome.

The chronological life span of specific yeast strains was determined by inoculating 5 ml of the designated medium with 10^6 cells, with a minimum of three replicates per strain. At the indicated times, samples were removed, diluted 100X (SD grown cells) or 300X (SEG grown cells) in sterile water, and 3 μl of the diluted cell suspension was added to 100 μl of sterile water prior to spreading on YPD plates. Colonies were counted after two days growth. All colonies were counted, regardless of size. During incubations, water was added to shaking cultures to maintain the 5 ml volume.

Nucleic acid techniques

All DNA manipulations were performed using standard techniques (Sambrook et al., 1989). Restriction and DNA modification enzymes were purchased from New England Biolabs unless otherwise noted. Plasmid DNA was prepared from *E. coli* by boiling lysis (Sambrook et al., 1989).

A null mutant and myc-tagged *INH1* were created using homologous recombination after transformation of PCR-generated DNA fragments. In the case of the *INH1* null mutant, genomic DNA template was prepared from the Open Biosystems strain carrying the KanMx4 cassette in place of the *INH1* gene (Longtine et al., 1998). Primers used

were 5′-CACAATTATGCCAATCAACTG-3′ (forward) and 5′-CTATCTTTCTATGCCTTTCCG-3′ (reverse). Plasmid pFA-13Myc-kanMX6 was used as PCR template for the Myc-tagged *INH1*. Primers used were AACGAAAGAAGATTGATTCTTTGGAAAATAAAATTGACTCGATGACCAAACGGATCCCCGGGTTAATTAA-3′ (forward) and 5′-ACCAGAGGAAAACTAGAATGATAGTTATCGAAAATGACAA AGCTCAGTGTGAATTCGAGCTCGTTTAAAC-3′ (reverse). Transformants resistant to geneticin (Sigma) were verified by PCR.

PCR primers for *ATP1* sequencing were 5′-CCATCTTTCCCATTGACGTT-3′ (forward) and 5′-CTTGCAGGCGATATTTCCTT-3′ (reverse). For cloning, *ATP1* alleles were PCR amplified from genomic DNA using Pfu Turbo DNA polymerase (Stratagene). Primers were 5′-CGGATTGGTACCTTTGGATCCAGAGC-3′ (forward) and 5′-GTGGGATCCTTTTCTAGAAGGGTTAT-3′ (reverse). PCR products were gel purified, digested with *Bam* HI and *Xba*I and ligated into pRS316 using T4 DNA ligase and transformed into *E. coli*. Restriction mapping identified recombinants. The resulting *ATP1*-containing plasmids (*pATP1* and *pATP1-111*) were transformed into the yeast strains TCY82 and BFY146.

Isolation of mitochondria, immuno-detection of proteins and measurement of F_1F_o-ATPase activity

Mitochondrial isolation was performed as described by Yaffe, 1991. Cells were grown in 1 liter of YPR to $OD^{600} = 1.5$. Mitochondrial yield was determined using the Coomassie Protein Assay (Pierce). Protein fractions were resolved on SDS-PAGE (Sambrook et al., 1989) and electroblotted onto nitrocellulose (BioRad) as previously described (Hanekamp and Thorsness, 1996). Inh1-myc was detected with a mouse monoclonal antibody (Sigma). Atp1p, Atp6p and Arg8 were detected using antisera generously provided by Dr. David Mueller (Lai-Zhang and Mueller, 2000), Dr. Jean Velours (Camougrand et al., 1995) and Dr. Thomas Fox (Steele et al., 1996), respectively. Signals were detected using the ECL detection method (Amersham). ATPase activities were determined using isolated mitochondria essentially as described (Tzagoloff, 1979). Reaction mixtures contained 120 μg of mitochondria and were incubated at 37°C for 12 minutes.

Measurement of relative ROS levels in yeast

2×10^7 cells from 24 hr SD cultures were washed 3X in 50 mM sodium phosphate, pH 7.4, and then incubated at room temperature for 20 minutes in 1 ml of the same buffer containing 0.05 mM 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) (Sigma). Cells were washed 3X with phosphate buffered saline, pH7.4. They were placed on a slide beneath an agarose pad that had been soaked in a 50:50 mixture of SD-complete and 2.5% 1,4-diazabicyclo(2,2,2)octane (Sigma). Cells were visualized using a Nikon Eclipse E 600 fluorescent microscope with a 60×1.4 numerical aperture oil-immersion objective, two separate channels (FITC and Texas red), and phase contrast. Wild-type PTY44 cells transformed with a plasmid encoding mitochondrially-targeted red fluorescent protein (gift of Dr. Janet Shaw) were similarity treated with H_2DCF-DA and fluorescence was visualized as above.

Results

Inh1p levels are independent of *YME1*

Mitochondria were prepared from WT and *yme1*Δ strains in which *INH1* was replaced by *INH1* that was C-terminally fused to a myc sequence recognized by anti-myc antibodies. Inh1-myc levels did not substantially vary in mitochondrial preparations that either contained or lacked Yme1p (Fig. 1). Similar results were obtained using extracts from whole cells instead of mitochondria. Thus Inh1p accumulation is not the basis for the inability of *yme1*Δ strains to grow in the absence of mtDNA.

Isolation and characterization of a dominant suppressor of the *yme1*Δ *inh1*Δ slow-growth phenotype

Yeast bearing *yme1*Δ and lacking mtDNA grow extremely slowly (Fig. 2). Extragenic suppressors of this phenotype have been isolated that serve to increase F_1-ATPase activity (Weber et al., 1995; Kominsky et al., 2002). Included in this class of suppressors are deletions of *INH1*, the structural gene that encodes the peptide Inh1p (Kominsky et al., 2002). We pursued the genetic analysis of this problem by screening *yme1*Δ *inh1*Δρ° yeast for spontaneous suppressors that would increase the growth rate on fermentable carbon sources. A single suppressed strain was isolated that carried

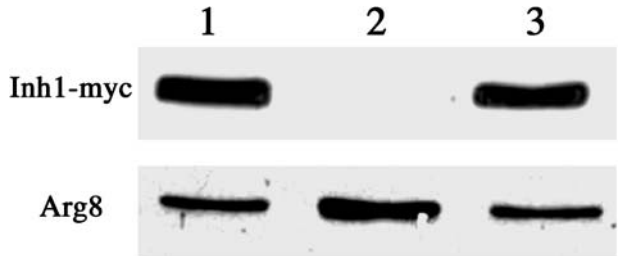

Fig. 1 Mitochondria were prepared from wild-type (PTY44), *INH1-myc* (BFY108), and *yme1*Δ *INH1-myc* (BFY109) ρ^+ strains grown in rich media with raffinose as the carbon source. 40 μg of mitochondrial proteins from each strain were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-myc and anti-Arg8 antibodies (mitochondrial control). Lane 1, *INH1-myc*; lane 2, WT; lane 3, *yme1*Δ *INH1-myc*

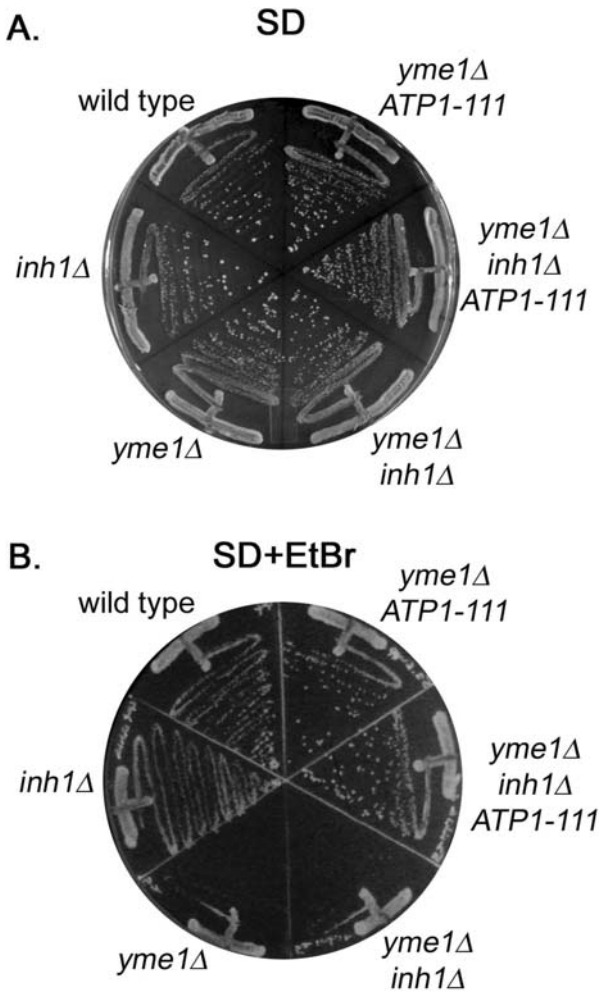

Fig. 2 Suppression of the *yme1*Δρ° and *yme1*Δ *inh1*Δ ρ° slow-growth phenotypes. Strains were streaked on (A) a complete synthetic glucose media (SD) and incubated for 3 days, or (B) or complete synthetic glucose media containing 25 μg/ml ethidium bromide (SD + EtBr) and incubated for 5 days at 30 °C. Strains: wild type, PTY44; *yme1*Δ, PTY52; *inh1*Δ, BFY101; *yme1*Δ *inh1*Δ, BFY107; *yme1*Δ *inh1*Δ *ATP1-111*, BFY137; *yme1*Δ *ATP1-111*, BFY138

a dominant suppressor of the *yme1*Δρ° slow-growth phenotype (Fig. 2). The suppressing activity was independent of the partial suppression afforded by *inh1*Δ (Fig. 2).

Crossing the *yme1*Δ strain containing the suppressor to a wild-type strain allowed isolation of a strain that contained just the suppressor. Testing for growth differences on plates between wild-type and suppressor-containing strains did not identify a phenotype that would clearly provide an effective screen to identify the suppressor. However, since strains with mutations in the *ATP3* and *ATP1* genes can suppress *yme1*Δ ρ° slow-growth (Weber et al., 1995; Kominsky et al., 2002) we examined the linkage of the new suppressor with previously isolated suppressors *ATP3-1* and *ATP1-75*. A strain bearing *yme1*Δ and the isolated suppressor was crossed with *yme1*Δ *ATP3-1* and spores from the resulting tetrads were tested for growth on media containing ethidium bromide to induce the loss of mtDNA (Materials and Methods). 16 out of 68 spores were not suppressed showing that the suppressor was not linked to *ATP3*. Similarly, a strain bearing *yme1*Δ and the isolated suppressor was crossed with a *yme1*Δ *ATP1-75* strain. In this case, all 72 spores were suppressed, thus demonstrating that the suppressor was an *ATP1* allele. Sequencing of the *ATP1* gene in the suppressor-containing and wild-type strains revealed a T for G substitution at position 331 in the mutant allele, producing the substitution of phenylalanine for valine at position 111 in Atp1p. This suppressing mutant allele was named *ATP1-111*. The valine at position 111 in yeast is highly conserved in eukaryotes and is also found in some bacteria. It is close in the amino acid sequence to the asparagine (residue 102) that is changed to isoleucine in the *ATP1-75* suppressor (Kominsky et al., 2002) (Fig. 3), but is not in a region of Atp1p that would be expected to be close to the site of Inh1p binding (Cabezon et al., 2003).

To confirm that this dominant *ATP1-111* mutation was responsible for the increased growth rate of *yme1*Δρ° strains, a *yme1*Δ strain was transformed with a plasmid containing either *ATP1*, *ATP1-111*, or no insert (Fig. 4). Transformation with the plasmid containing *ATP1-111* suppressed the ρ° slow-growth phenotype, as expected for a dominant mutation, but no suppression was observed when the plasmid contained either the wild-type *ATP1* or lacked an insert. Similarly, plasmid borne *ATP1-111* was able to suppress the ρ° slow-growth phenotype of a *yme1*Δ *atp1*Δ strain.

The V111F mutation in Atp1p increases the ATPase activity of *ATP1-111* strains compared to strains with WT Atp1p

Suppressors of the *yme1*Δρ° slow-growth phenotype, such as *ATP1-75* and *ATP3-1*, have previously been shown to have increased F_1-ATPase activity compared with wild-type and *yme1*Δ yeast (Kominsky et al., 2002). F_1-ATPase activity in mitochondria isolated from ρ^+ *yme1*Δ *ATP1-111* was also increased compared to wild-type and *yme1*Δ strains (Fig. 5A). The relative concentration of Atp1p in wild-type and suppressed strains was assessed by Western blot and no significant change in the concentration of Atp1p was evident (Fig. 5B).

The V111F mutation in Atp1p does not increase ρ° growth in the absence of Atp2p or Atp3p

The equivalent residue to valine 111 of *S. cerevisiae* Atp1p in bovine F_1-ATPase is valine 117 of the α-subunit. The position of Val117 in the bovine F_1-ATPase structure suggested that the effect of the yeast Atp1p V111F suppressing

A. α -subunit/Atp1p

S. cerevisiae	98	GMALNLEPGQVGIVLFGSDRLVKE-G
S. cerevisiae ATP1-111	98	GMALNLEPGQVGI**F**LFGSDRLVKE-G
S. cerevisiae ATP1-75	98	GMAL**I**LEPGQVGIVLFGSDRLVKE-G
Bos taurus	104	GMSLNLEPDNVGVVVFGNDKLIKE-G
Escherichia coli	61	AIALNLERDSVGAVVMGPYADLAE-G
Bacillus subtilis	61	GMAQNLEESNVGIVILGPFSEIRE-G
Sulfolobus acidocaldarius	103	FVARGVSIPALDRQTKWHFVPKVKSG
Halobacterium salinarum	107	FLDRGVDAPGIDLLDTDWEFEPTVEAG
Methanosarcina barkeri	81	GIAFNVDEKEIGTVLLGEYSHLHA-G

B. γ -subunit/Atp3p

S. cerevisiae	292	RQAVITNELVDIITGASSLG	311
S. cerevisiae ATP3-1	292	RQAVI**A**NELVDIITGASSLG	311
S. cerevisiae ATP3-5	292	RQAVITNELVD**T**ITGASSLG	311
Bos taurus	279	RQAVITKELIEIISGAAAL	297
Spinacia oleracea	345	RQAKITGEILEIVAGANACV	364
Escherichia coli	269	RQASITQELTEIVSGAAAV	287

Fig. 3 Amino acid sequences of ATP synthase α and γ subunits. (A) Amino acid sequence for ATP synthase α-subunits (Atp1p) in the segment that contains the mutations in *ATP-111* and *ATP1-75*. Database identifiers for α-subunits: Sc NP_009453; Bt AAA30399; Ec AAA24735; Bs CAA82258; Sa AAA72192; Hs CAA49775; Mb AAC38056. (B) C-terminal amino acid sequences of ATP synthase γ-subunits (Atp3p). Database identifiers for γ-subunits: Sc NP_009595; Bt AAA30398; So CAA53734; Ec AAA24736. Mutations in *ATP1-111*, *ATP1-75*, *ATP3-1* and *ATP3-5* are in bold

mutation is due to a change in the interaction of Atp1p with Atp2p and/or Atp3p. Ideally, the requirement for intact *ATP2* and/or *ATP3* genes to be present to mediate the effect of the *ATP1-111* suppressor should be assessed in a strain lacking *YME1*. Previous work has demonstrated that *yme1*Δ *atp2*Δρ° and *yme1*Δ *atp3*Δ ρ° strains were inviable (data not shown). Similarly, *yme1*Δ *atp2*Δ *ATP1-111*ρ° and *yme1*Δ *atp3*Δ *ATP1-111* ρ° strains were inviable (data not shown), indicating that both Atp2p and Atp3p are required to mediate the suppressing effects of *ATP1-111* on the *yme1*Δρ° slow-growth phenotype. The *ATP1-111* allele was unable to rescue the petite-negative phenotypes of *atp3*Δ (Fig. 6A) or *atp2*Δ (Fig. 6B) yeast, as assessed by growth on media containing ethidium bromide.

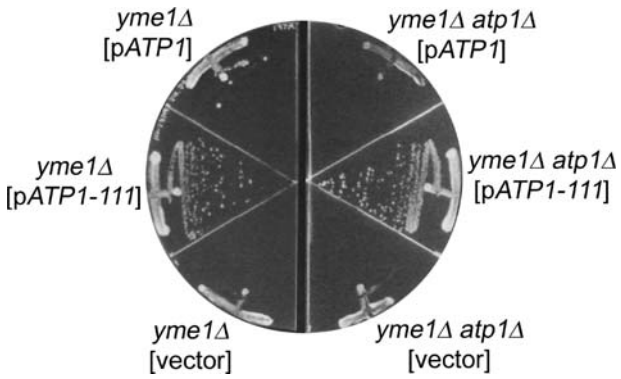

Fig. 4 Cloned *ATP1-111* rescues the slow-growth phenotype of *yme1*Δ strains. *yme1*Δ and *yme1*Δ *atp1*Δ strains were transformed with either empty vector ([vector], pRS316), the vector bearing wild-type *ATP1* ([p*ATP1*]), or the vector bearing *ATP1-111* ([p*ATP1-111*]). Transformed cells were grown on synthetic glucose media containing 25 μg/ml ethidium bromide and incubated for 5 days at 30°C to compare growth. Strains: *yme1*Δ, TCY82; *yme1*Δ *atp1*Δ, BFY146

The V111F mutation in Atp1p suppresses the ρ° slow-growth phenotype of *atp4*Δ and *atp5*Δ yeast

Crosslinking experiments have demonstrated interactions of the Atp4p subunit of F_o, with the Atp1p and Atp2p subunits of F_1 in yeast (Enns and Criddle, 1977; Todd and Douglas, 1981; Soubannier et al., 1999), and in *E. coli*, cross-linking is observed between Cys90 in the N-terminal domain of the α subunit of F_1-ATPase (equivalent to yeast Atp1p) and a Cys at the C-terminus of subunit b (equivalent to Atp4p) (Rodgers and Capaldi, 1998). Consequently, it was possible that the V111F mutation in Atp1p altered its interaction with Atp4p. Growth of *atp4*Δ *ATP1-111* ρ° and *yme1*Δ *atp4*Δ *ATP1-111* ρ° strains was compared with the corresponding *atp4*Δ ρ° and *yme1 atp4*Δ ρ° strains. When cultured on media that induced the loss of mtDNA, *atp4*Δ *ATP1-111* strains grew faster than wild-type or *atp4*Δ strains (Fig. 6C) and *yme1*Δ *atp4*Δ *ATP1-111* strains grew faster than *yme1*Δ *atp4*Δ strains (Fig 6D). These results showed that the suppressive effects of *ATP1-111* were not dependent on Atp4p.

The oligomycin sensitivity conferring protein (OSCP) subunit of bovine ATP synthase (equivalent to Atp5p in yeast) binds to the N-terminus of the α subunit (equivalent to Atp1p) (Xu et al., 2000). Previous work has established that a null mutation in *ATP5* eliminates the oligomycin-sensitive ATPase activity, uncouples F_1 from subunits of F_o, and produces a high level of mtDNA instability (Uh et al., 1990; Prescott et al., 1994). Another possibility was that the *ATP1-111* allele altered the interaction between Atp1p and Atp5p. *atp5*Δ *ATP1-111* ρ° yeast grew faster than *atp5*Δ ρ° yeast (Fig. 6E), and *yme1*Δ *ATP1-111 atp5*Δ ρ° yeast grew much faster than *yme1*Δ *atp5*Δ ρ° yeast (Fig. 6F). Thus, the suppressive effect of *ATP1-111* with respect to

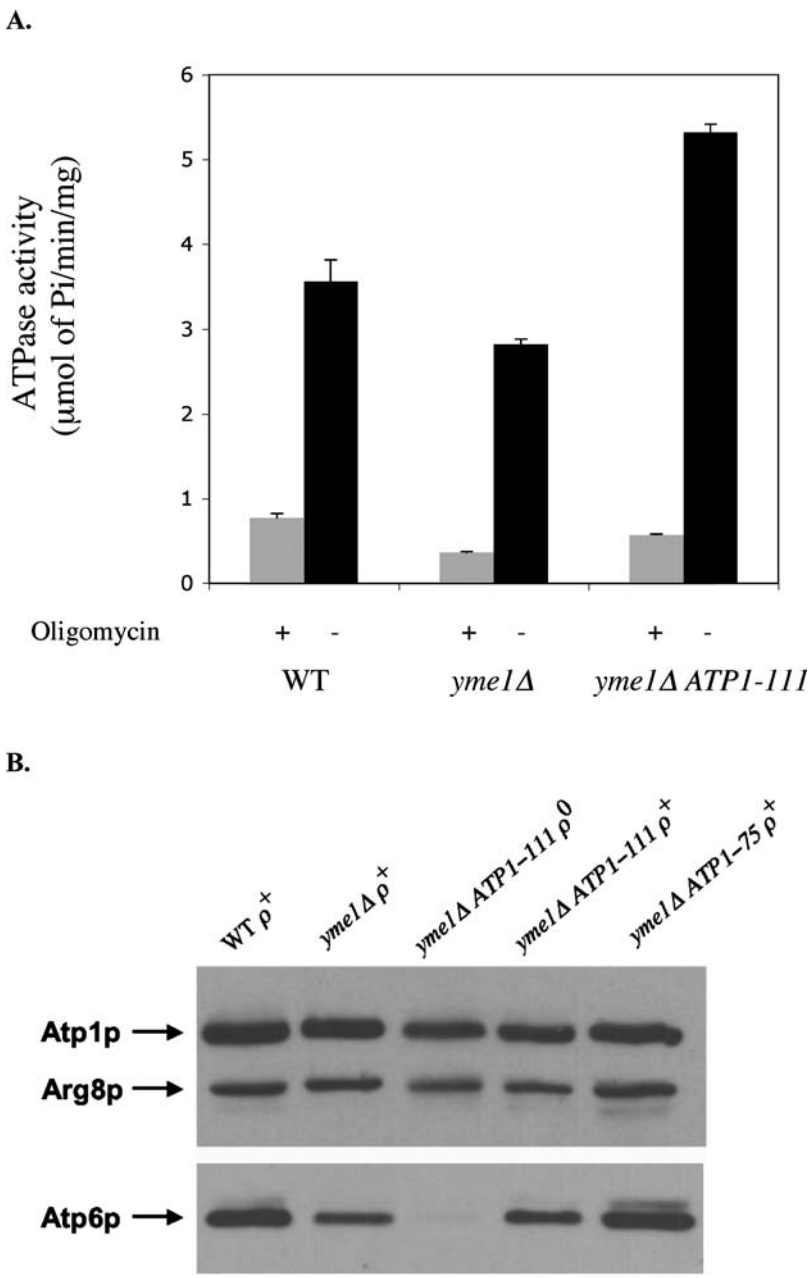

Fig. 5 F_1F_o-ATPase activity in isogenic yeast. (A) ATPase activity in isolated mitochondria. Reactions for each strain were performed in triplicate with (+) or without (−) oligomycin, an inhibitor of F_1F_o-ATPase activity. F_1F_o-ATPase activity is the difference between the activities measured in the absence and presence of oligomycin. F_1F_o-ATPase activity in mitochondria isolated from *yme1*Δ *ATP1-111* was statistically significantly different from those from WT and *yme1*Δ (Student's *t*-test at the level of $p < 0.0005$). Data are means +/− standard error of the mean. Strains used were the following: WT (wild type), PTY44; *yme1*Δ, PTY52; *yme1*Δ *ATP1-111*, BFY138. (B) Western blot of isolated mitochondrial proteins using rabbit polyclonal antibodies against Atp1p, Arg8p (matrix protein control) and Atp6p (F_o protein control). Proteins were separated on an SDS-PAGE gel, transferred to nitrocellulose, and probed with antibodies. Strains: wild type ρ^+(WT), PTY44; *yme1*Δ ρ^+, PTY52; *yme1*Δ *ATP1-111* ρ°, BFY138; *yme1*Δ *ATP1-111* ρ^+, BFY138; *yme1*Δ *ATP1-75* ρ^+, PTY93

slow growing *yme1*Δ ρ° yeast did not involve an altered interaction with Atp5p.

Growth characteristics of yeast bearing *ATP1* and *ATP3* suppressing mutations

In many cases, ρ° and ρ^+ strains bearing suppressing mutations in *ATP1* and *ATP3* grew on agar plates at rates quite similar to the isogenic wild-type strain. As these alleles of *ATP1* and *ATP3* had changes in highly conserved residues and were thus expected to significantly impact growth, we pursued a more detailed study of growth rates during logarithmic phase and yields in liquid culture. In contrast to wild-type ρ° yeast, which grew more slowly than wild-type ρ^+, the growth rate of *ATP1-111* ρ° yeast approached that of a wild-type ρ^+ yeast on comparable media (Table 2). ρ° yeast bearing *ATP1-75*, *ATP3-1*, or *ATP3-5* suppressing mutations also grew faster than wild-type ρ° yeast.

Yeast bearing suppressing mutations in *ATP1* and *ATP3* grew noticeably slower on non-fermentable carbon sources than isogenic wild-type yeast (Table 2, YPEG and SEG-complete). This negative impact on growth in media that requires respiration may reflect decreased coupling of the F_1

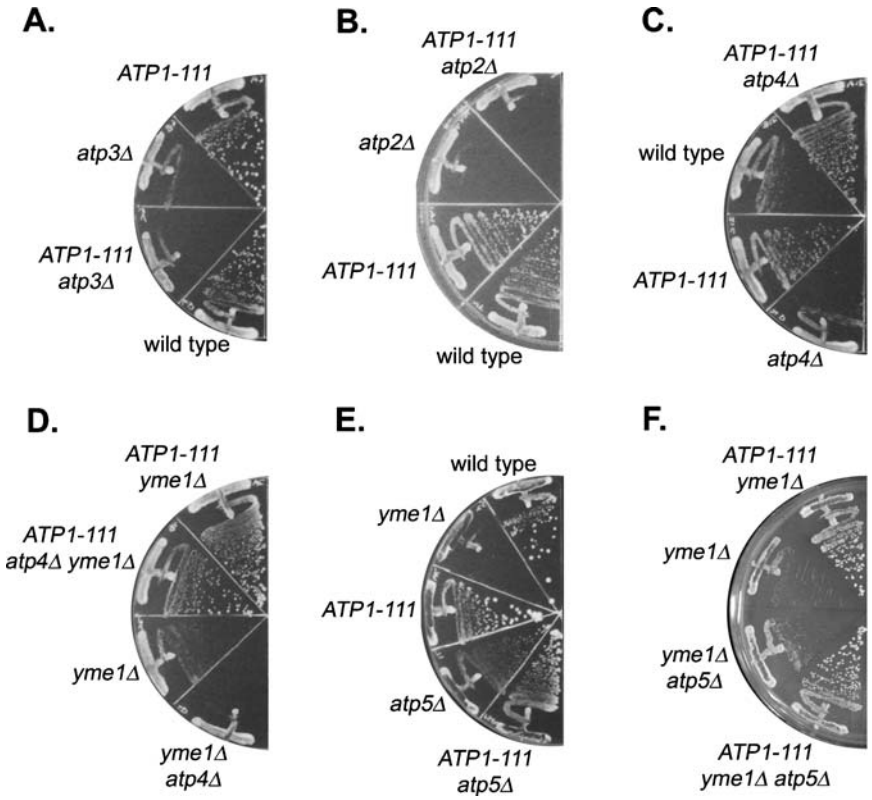

Fig. 6 Requirement of F_1 and F_o subunits for *ATP1-111* mediated suppression of ρ° slow-growth phenotypes. Yeast were streaked on synthetic glucose plates containing 25 μg/ml ethidium bromide and incubated for 5 days at 30°C. (A) *ATP1-111* does not suppress the *atp3*Δ ρ° slow-growth phenotype. Strains: *ATP1-111*, BFY141; *atp3*Δ, TCY37; *ATP1-111 atp3*Δ, BFY148; wild type, PTY44. (B) *ATP1-111* does not suppress the *atp2*Δ ρ° slow-growth phenotype. Strains: *ATP1-111 atp2*Δ, BFY178; *atp2*Δ, TCY48; *ATP1-111*, BFY141; wild type, PTY44. (C) *ATP1-111* suppresses the *atp4*Δ ρ° slow-growth phenotype. Strains: *ATP1-111 atp4*Δ, BFY152; wild type, PTY44; *ATP1-111*, BFY141; *atp4*Δ, DKY40. (D) *ATP1-111* suppresses the *atp4*Δ *yme1*Δ ρ° slow-growth phenotype. Strains: *ATP1-111 yme1*Δ, BFY138; *yme1*Δ *atp4*Δ, *ATP1-111*, BFY160; *yme1*Δ, PTY52; *yme1*Δ *atp4*Δ, DKY48. (E) *ATP1-111* suppresses the *atp5*Δ ρ° slow-growth phenotype. Strains: wild type, PTY44; *yme1*Δ, PTY52; *ATP1-111*, BFY141; *atp5*Δ, TCY71; *ATP1-111 atp5*Δ, BFY149. (F) *ATP1-111* suppresses the *atp5*Δ *yme1*Δ ρ° slow-growth phenotype. Strains: *ATP1-111 yme1*Δ, BFY138; *yme1*Δ, PTY52; yme1Δ atp5Δ, BFY190; *ATP1-111 yme1*Δ *atp5*Δ, BFY188

and F_o subunits of ATP synthase in conditions requiring ATP synthesis. In contrast, these same suppressors had little or no effect on growth of ρ^+ strains by fermentation (Table 2, YPD and SD-complete). Wild-type ρ^+ growth is substantially faster than wild-type ρ° growth on fermentable media (Table 2) due to the presence of F_o, which provides a growth advantage due to the ability of ρ^+ cells to generate a membrane potential by coupled H^+ translocation and ATP hydrolysis. Deletion of *CAT5*, a ubiquinone biosynthesis gene, precludes electron transport from contributing to membrane potential in mitochondria, but unlike the loss of mtDNA, the formation of coupled F_1F_o-ATPase is not impaired. *cat5*Δ and *cat5*Δ *ATP1-111* ρ^+ yeast (and related double mutants) have the same growth rate as wild-type ρ^+ yeast on fermentable media (Table 2, SD-complete). Therefore, loss of the electron transport process that remains under glucose repression does not significantly impact growth by fermentation, demonstrating the critical importance of proton pumping by F_1F_o-ATPase in establishing the membrane potential under these conditions.

Yields of several strains were measured by absorbance at 600 nM and by numbers of colony forming units (CFU) (Table 3). OD^{600} values for strains grown in synthetic glucose media (SD) reached a maximum after 3 days, but those for strains grown in synthetic ethanol/glycerol media (SEG) continued to increase up to 6 or 8 days. The CFU/OD^{600} ratios of yeast grown in SD revealed that *ATP3-5* mutants were dying more rapidly than the other strains. Of the strains grown in SEG, wild-type ρ^+ yeast showed a greater decrease in CFU/OD^{600} (∼40%) than *ATP1-111*, *ATP1-75*, or *ATP3-1* (∼25%) ρ^+ yeast. Therefore, wild-type ρ^+ yeast died more rapidly than strains bearing suppressing mutations in *ATP1* and *ATP3*. CFU/OD^{600} ratios for *yme1*Δ *ATP1-75* and *yme1*Δ *ATP3-1* increased over the same period in SEG-complete, indicating extended cell viability, whereas *yme1*Δ and *yme1*Δ *ATP1-111*

Table 3 Yields of ρ^+ cells grown in SD-complete and SEG-complete media

Strain name	Relevant genotype	SD-complete Day 3 [a]CFU/OD^{600}	SEG-complete Day 3 CFU/OD^{600}	SEG-complete Day 6 CFU/OD^{600}	SEG-complete Day 8 CFU/OD^{600}
PTY44	WT	465	215	125	130
BFY141	*ATP1-111*	460	200	165	150
KWY94	*ATP1-75*	525	190	195	140
KWY96	*ATP3-1*	420	195	180	150
PTY100	*ATP3-5*	345			
PTY52	*yme1*Δ	310	165	160	135
BFY138	*yme1*Δ *ATP1-111*	355	170	160	140
PTY102	*yme1*Δ *ATP1-75*	360	130	155	185
PTY109	*yme1*Δ *ATP3-1*	275	120	185	185
KWY91	*yme1*Δ *ATP3-5*	115			

[a]CFU = colony forming unit; OD^{600} = optical density of culture at 600 nm.

ratios decreased, indicating faster rates of death than growth.

Chronological life span of strains containing mutations in *ATP1* and *ATP3*

Because the culture yield results (Table 3) indicated that wild-type ρ^+ strains died more rapidly in SEG than ρ^+ strains with mutations in *ATP1* and *ATP3*, a more detailed study of these yeast strains' chronological life span (CLS) was undertaken. The assay for CLS has usually been conducted in SD media (Longo et al., 1997; Mazzoni et al., 2005), following the survival of yeast cells into stationary phase, although after the glucose has been used up yeast will undergo diauxic shift to growth on ethanol. As expected, CLS for *ATP1–111*, *ATP1-75*, and *ATP3-1* strains (50% viability after ∼11.5–12.5 days) was longer than wild-type strains (50% viability after ∼10 days) (Fig. 7A). The short CLS for *ATP3-5* yeast (50% viability in ∼4 days) may have been due to an inability of strains bearing this respiration-negative mutation to generate ATP *via* oxidative phosphorylation. Similarly, ρ^+ strains bearing *cat5*Δ are incapable of respiration and had a much shorter CLS (50% viability after ∼1.5 days) than wild-type ρ^+ or *ATP3-5* ρ^+ yeast (Fig. 7A). *yme1*Δ ρ^+ yeast also had a shorter CLS (50% viability after ∼7 days) than wild type ρ^+ strains, but double mutant ρ^+ strains bearing *yme1*Δ and a suppressing mutation in *ATP1* or *ATP3* had even shorter CLS (50% viability after ∼5–6 days) than *yme1*Δ strains (Fig. 7B). Therefore, the increase in CLS produced by the suppressing mutations alone was reversed in the presence of *yme1*Δ.

The CLS for wild-type ρ°, and ρ° yeast strains bearing suppressor mutations were compared, taking the numbers of colony forming units after one day of incubation as 100% viability (Fig. 8). Particularly striking was the reversal of relative CLS for ρ^+ and ρ° versions of wild-type and suppressor bearing yeast strains. Wild-type ρ° yeast aged more slowly (50% viability after ∼2.5 days) than ρ° strains containing suppressors (50% viability after ∼1.7–1.9 days). In contrast to the greatly reduced CLS of *cat5*Δ ρ^+ yeast compared to wild-type ρ^+ yeast, CLS for *cat5*Δ ρ° yeast was similar to wild-type ρ° yeast.

Reactive oxygen species in ρ^+ and ρ° strains

Cell death during chronological aging in yeast is associated with apoptotic markers (Herker et al., 2004) and apoptosis is associated with the generation of reactive oxygen species (ROS) in mitochondria (Madeo et al., 1999; Fleury et al., 2002; Temple et al., 2005). Therefore, some of the strains that had short CLS were tested for their production of ROS relative to that generated in a wild-type ρ^+ strain using the fluorescent probe, H_2DCF-DA (Jakubowski and Bartosz, 1997) (Fig. 9). Wild-type ρ° and *cat5*Δρ° cultures had higher percentages of cells that exhibited ROS-dependent fluorescence than did the wild-type and *ATP3-5* ρ^+ cultures. The percentage of cells exhibiting ROS-dependent fluorescence in cultures of the *cat5*Δρ^+ strain was not significantly different from those of either the ρ° or the other ρ^+ cultures. Wild-type ρ^+ and ρ° cells were transformed with a plasmid carrying mitochondrially-targeted red fluorescent protein and treated with H_2DCF-DA. Co-localization of ROS fluorescence and mitochondrial fluorescence was observed in both cases (Fig. 10). The fluorescent signals from H_2DCF-DA or mitochondrially-targeted red fluorescent protein were specifically dependent upon inclusion of the reagent in the cells examined.

Spore viability of strains containing mutations of *YME1*, *ATP1*, and *ATP4*

Because sporulation is a respiration dependent process (Croes, 1967; Gorsich and Shaw, 2004) we anticipated that *YME1* and *ATP1* mutant alleles would have decreased sporulation. However, respiring diploids containing homozygous and heterozygous combinations of mutant alleles

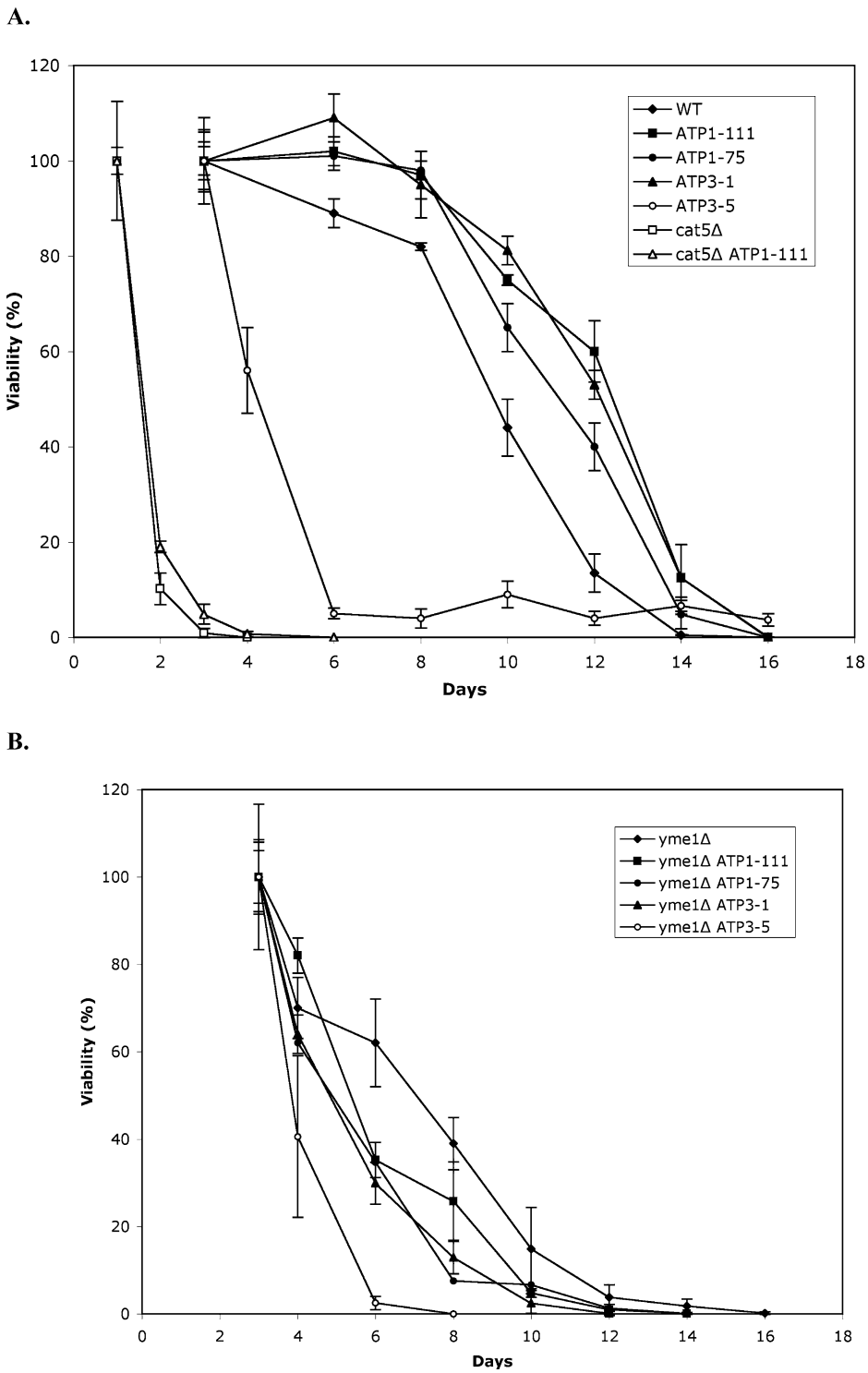

Fig. 7 Chronological life span of ρ^+ strains in synthetic glucose media at 30°C. Colony forming units (CFU) were calculated as the mean of triplicate experiments for each strain. CFU measurements were converted to % viability taking the CFU after 3 days of culturing as 100% viability, except for the strains bearing *cat5*Δ CFU after 1 day was taken as 100% viability. Data are means +/− standard error of the mean. (A) Strains: wild type, PTY44; *ATP1-111*, BFY141; *ATP1-75*, KWY94; *ATP3-1*, KWY96; *ATP3-5*, PTY100; *cat5*Δ, TCY1; *cat5*Δ *ATP1-111*, KWY116. (B) Strains: *yme1*Δ, PTY52; *yme1*Δ *ATP1-111*, BFY138; *yme1*Δ *ATP1-75*, PTY93; *yme1*Δ *ATP3-1*, PTY109; *yme1*Δ *ATP3-5*, KWY91

appeared to sporulate as well as the isogenic wild-type diploid. Because the chronological life span of haploid yeast containing mutant alleles of *YME1* and *ATP1* were altered, we examined the ability of dissected spores bearing *ATP1-111* or *atp1*Δ to germinate as a function of time on sporulation medium (Table 4). *atp4*Δ was also included in some crosses as the consequence of this mutation is the loss of a functional F_o portion of the mitochondrial ATP synthase. Tetrads from different crosses were dissected to YPD 5, 15, and 40 days after transfer of diploids to sporulation media, and characterized as complete (all four spores germinating), incomplete (1, 2, or 3 spores germinating), or no viable spores. The complete tetrads were subdivided according to how many spores produced respiration competent colonies.

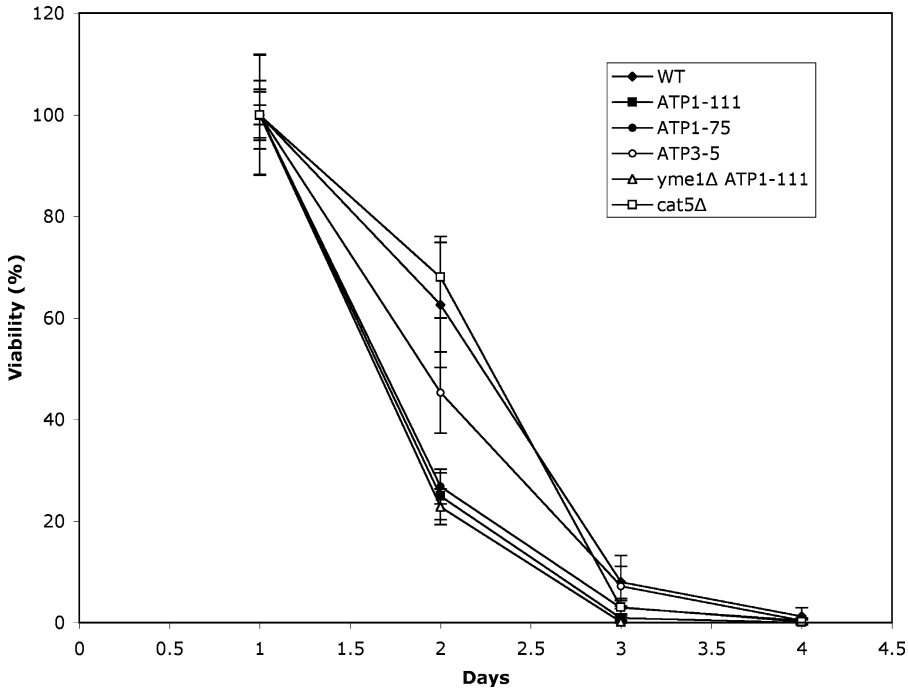

Fig. 8 Chronological life span of ρ° strains in SD-complete at 30°C. Colony forming units (CFU) were calculated as the mean of triplicate experiments for each strain. All CFU measurements were converted to % viability taking the CFU after 3 days of culturing as 100% viability. Data are means +/− standard error of the mean. ρ° strains: WT (wild type), PTY44; *ATP1-111*, BFY141; *ATP1-75*, KWY94; *ATP3-1*, KWY96; *ATP3-5*, PTY100; *yme1*Δ *ATP1-111*, BFY138; *cat5*Δ, TCY1

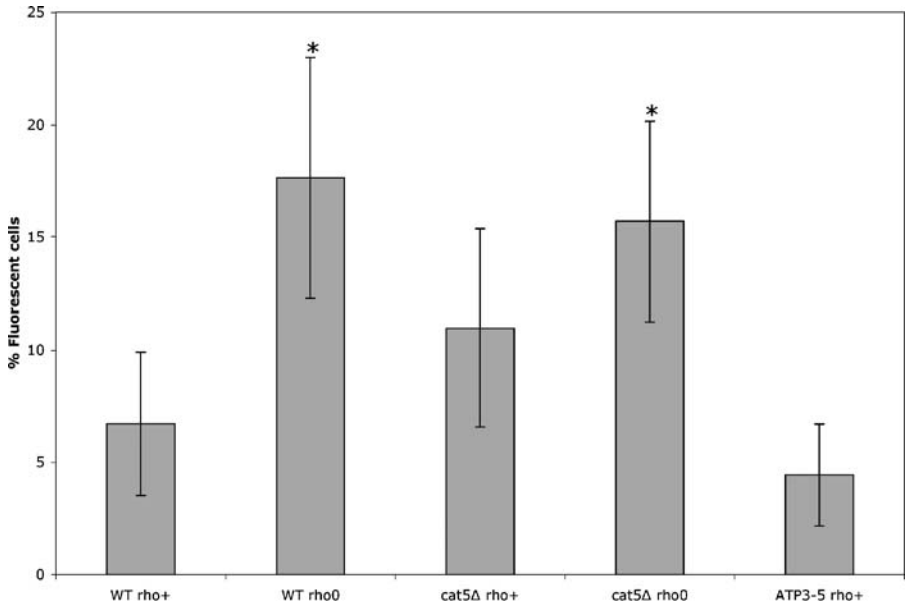

Fig. 9 Cell-based assay of ROS in yeast. Strains were incubated with H_2DCF-DA and those cells displaying increased fluorescence were counted as positive. A minimum of 100 cells for each strain were examined blind by three separate individuals. Samples marked with "*" indicates a statistically significant difference (Student's *t*-test, $p < 0.05$) from WT ρ^+. Strains: WT rho+, PTY44; WT rho0, PTY44 lacking mtDNA; *cat5*Δ rho+, TCY1; *cat5*Δ rho0, TCY1 lacking mtDNA; *ATP3-5* rho+, PTY100

As a baseline for the comparison, the dissected spores from a homozygous wild-type diploid (WT × WT) had 2, 5 and 21% incomplete tetrads after 5, 15, and 40 days incubation, respectively. All of the complete tetrads from WT × WT produced four respiration competent colonies, and the large majority (28/33; 85%) of the incomplete tetrads after 40 days had three colonies that could respire. Therefore, in these incomplete tetrads one of the spores failed to germinate but the other three were unaffected with respect to respiratory growth. Spores derived from heterozygous *ATP1-111* diploids experienced no decrease in viability, but *ATP1-111* homozygous diploids gave rise to a greater population of incomplete tetrads after 40 days (35%) than tetrads containing at least one copy of wild-type *ATP1* (∼21%).

Because of the different mechanisms that F_1F_o-ATPase and F_1-ATPase use to establish a mitochondrial membrane potential and hence a viable mitochondrial compartment (proton pumping versus promoting electrogenic transport), we chose to examine the spore viability of yeast bearing defective F_1F_o-ATPase and F_1-ATPase. To perturb the F_o portion of ATP synthase, *atp4*Δ mutations were introduced into the cross as yeast strains bearing *atp4*Δ are both

Table 4 Viability of spores from crosses involving WT and strains containing *atp4*Δ, *atp1*Δ, *ATP1-111*, and *yme1*Δ

Cross	Number of days on SPO plate	Number of days on SPO plate: Complete tetrads: 4YPEG spores %	3YPEG spores %	2YPEG spores %	1YPEG spore %	0YPEG spores %	Incomplete tetrads %	No viable spores %	Total tetrads
WT × WT	5	96	0	0	0	1[a]	2	0	84
(PTY33 × PTY44)	15	95	0	0	0	0	5	0	58
	40	78	0	0	0	0	22	0	153
WT × *ATP1-111*	5	100	0	0	0	0	0	0	65
(PTY44 x BFY142)	15	90	0	0	0	0	8	2	60
	40	79	0	0	0	0	21	0	62
ATP1-111 × *ATP1-111*	5	92	0	0	0	3	5	0	66
(BFY141 × BFY142)	15	95	0	0	0	0	5	0	88
	40	63	2	1	0	0	35	0	110
WT × *atp4*Δ	5	0	0	83[b]	0	10[b]	6	1	131
(PTY44 × DKY41)	15	0	0	82	0	9	8	1	119
	40	0	0	74	0	0	26	0	62
ATP1-111 × *atp4*Δ	5	0	0	72[b]	0	24[b]	4	0	263
(BFY141 × DKY41)	15	0	0	74	0	17	8	1	124
	40	0	0	47	0	12	38	3	58
WT × *atp1*Δ *atp4*Δ	5	0	0	15	61	11	14	0	66
(PTY33 × BFY165)	15	0	0	11	58	18	9	3	88
	40	0	0	9	34	11	44	3	110
ATP1-111 × *atp1*Δ *atp4*Δ	5	0	0	15	50	28	7	0	60
(BFY142 × BFY165)	15	0	0	15	53	20	5	7	60
	40	0	0	2	7	1	55	35	110
WT × *yme1*Δ	5	95	0	0	0	0	5	0	40
(PTY33 × PTY52)	14	82	0	0	0	0	18	0	66
	40	37	1	1	0	0	58	4	176
*yme1*Δ × *yme1*Δ	5	93	2	0	0	2	3	0	59
(PTY52 × PTY60)	15	68	3	0	0	3	25	0	60
	40	40	0	0	0	0	56	4	110
*yme1*Δ × *yme1*Δ *atp4*Δ	5	0	0	88	0	2	11	0	66
(BFY156 × DKY48)	15	0	0	80	0	0	20	0	66
	40	0	0	14	0	0	78	8	93
*yme1*Δ *ATP1-111* × *yme1*Δ *atp4*Δ	5	0	0	78[b]	0	0	22	0	40
(BFY140 × DKY48)	40	0	0	5[b]	0	0	20	75	60
*yme1*Δ × *yme1*Δ *ATP1-111*	5	77	10[c]	0	0	0	11	1	88
(PTY52 × BFY139)	15	54	12[c]	2[c]	0	0	31	1	88
	40	15	3	2	0	0	76	4	110
*yme1*Δ *ATP1-111* ×	5	67	15[c]	3[c]	0	3[c]	10	1	88
*yme1*Δ *ATP1-111*	15	65	11[c]	0	2[c]	9[c]	13	0	88
(BFY138 × BFY139)	40	14	3	1	0	0	73	8	154
*yme1*Δ *ATP1-75*	5	52	17[c]	3[c]	2[c]	23[c]	2	0	87
× *yme1*Δ *ATP1-75*	15	58	9[c]	9[c]	2[c]	17[c]	3	1	88
(PTY93 × PTY102)	40	11	6	0	0	2	67	5	88

[a] Four very slow growing colonies.
[b] Spores containing *atp4*Δ and not growing on YPEG were ρ^-/ρ°.
[c] Spores not growing on YPEG were ρ^-/ρ°

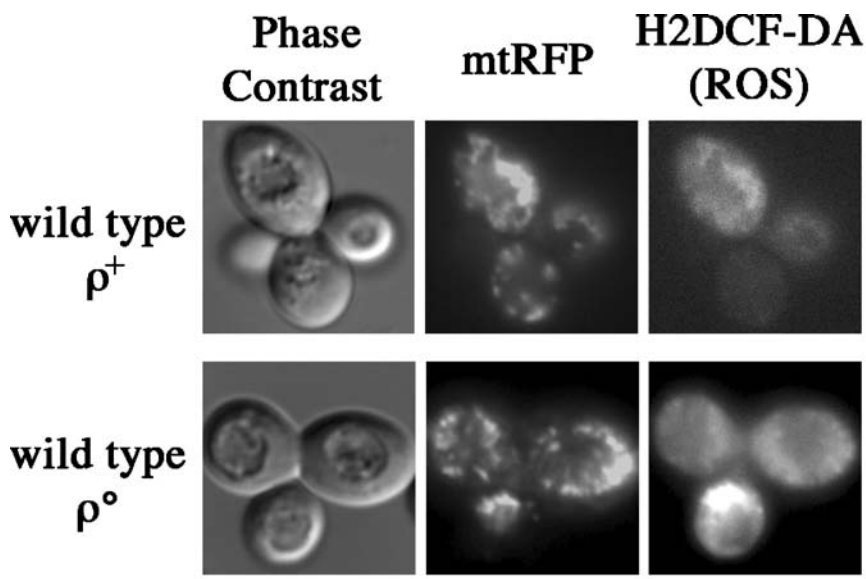

Fig. 10 Cellular location of ROS generation in yeast cells. Wild type ρ^+ (PTY44) and wild type ρ° (PTY44 ρ°) transformed with a plasmid expressing mitochondrially-targeted red fluorescent protein (mtRFP) were incubated with H_2DCF-DA. Cells were observed in a fluorescence microscope using excitation/emission filters that allowed detection of ROS-dependent fluorescence of H_2DCF-DA and mtRFP

respiratory incompetent and rapidly become ρ^- or ρ°, presumably as the direct result of the lack of the F_o portion of the mitochondrial ATP synthase (Velours et al., 1987; Paul et al., 1989). The F_1 portion of ATP synthase was perturbed in crosses through the use of *ATP1-111* and *atp1*Δ alleles. Spore viability was not significantly impacted in tetrads from heterozygous *atp4*Δ strains, but, unexpectedly, there was a significant number of spores that were genetically wild type for *ATP4* but were respiration incompetent due to the presence of ρ^- or ρ° mitochondria. This phenomenon was exacerbated when *ATP1-111* or *atp1*Δ was present in the cross. When sporulation time for WT × *atp4*Δ increased to 40 days, no complete tetrads were found with spores that were *ATP4* ρ^-/ρ°, indicating that spores with this genotype were becoming inviable during the prolonged incubation. In contrast, prolonged incubation of spores from the corresponding *ATP1-111* × *atp4*Δ cross did not result in loss of these spores, indicating that they were stabilized by the *ATP1-111* mutation. When the *atp1*Δ allele was also present in these crosses there was a substantial decrease in spore viability as spores aged. A particularly informative cross was *ATP1-111* × *atp4*Δ *atp1*Δ. After 40 days, only 10% of the tetrads produced four viable spores. 55% were incomplete and 35% had no viable spores. This result again demonstrated that *ATP1-111* decreased the long-term viability of spores compared to *ATP1*. The distribution of the 154 spores that germinated from 110 40-day tetrads of *ATP1-111* × *atp4*Δ *atp1*Δ was: *atp4*Δ *atp1*Δ = 22%, *ATP1-111 atp4*Δ = 21%, *ATP4 atp1*Δ = 16%, *ATP4 ATP1-111* = 40%. Viable spores of all possible genotypes were found, but those spores bearing *atp4*Δ or *atp1*Δ had a clear disadvantage upon aging. Overall, there were two effects of replacement of *ATP1* by *ATP1-111*: generally decreased long-term spore viability, and increased viability of *ATP4*-containing, ρ^-/ρ° spores.

The presence of either one or two copies of the *yme1*Δ allele had a profound effect on the viability of aged spores, leading to a 2.5-fold increase in the number of incomplete tetrads after 40 days of incubation on sporulation media compared to tetrads from a homozygous wild-type diploid. However, there was no correlation between a spore being viable/inviable and the presence of the *yme1*Δ allele in the spore itself. The inclusion of either *ATP1-111* or *ATP1-75* alleles capable of suppressing the petite-negative phenotype of *yme1*Δ in the cross not only was unable to suppress the decreased spore viability but actually exacerbated the decreased viability of aged spores (compare *yme1*Δ × *yme1*Δ to *yme1*Δ *ATP1-111* × *yme1*Δ *ATP1-111* at 40 days, Table 4). Interestingly, the inclusion of the heterozygous *atp4*Δ and *ATP1-111* in a homozygous *yme1*Δ cross led to massive spore inviability as 75% of all tetrads had no viable spores after 40 days.

Discussion

The bioenergetic basis for petite-negativity

The process of membrane potential generation in ρ° yeast cells is dependent upon Yme1p, an ATP and metal dependent AAA-protease imbedded in the inner mitochondrial membrane with the active sites located in the intermembrane space (Kominsky et al., 2002). Previous work indicated partial suppression of *yme1*Δ ρ° slow-growth phenotype by disrupting *INH1*, the structural gene for the endogenous peptide inhibitor of F_1-ATPase, Inh1p. As judged by immunoblots of total Inh1p in yeast cells and mitochondria, the concentration of Inh1p is not discernibly altered in yeast lacking Yme1p. We hypothesized that there are multiple substrates of Yme1p

that accumulate in *yme1*Δ strains and inhibit F_1-ATPase. We chose to look for other inhibitors in *yme1*Δ *inh1*Δ ρ° strains as they still were slower-growing than wild-type ρ° yeast, reasoning that mutation of additional F_1-ATPase inhibitors would increase the growth of these ρ° yeast. Suppressors of the *yme1*Δ *inh1*Δ ρ° growth defect were very rare ($<1 \times 10^{-8}$ events/cell division). The characterized suppressor in this screen was found to be dominant and independent of *inh1*Δ. Thus, Inh1p is only a minor factor in creating the petite-negative phenotype of *yme1*Δ strains. Consequently, we conclude that the role Yme1p plays in leading to the appropriate level of F_1-ATPase activity is not through determining the concentration of the inhibitor, Inh1p.

This newly isolated suppressor of the *yme1*Δ ρ° slow-growth phenotypes has a phenylalanine for valine change at position 111 of Atp1p, the α-subunit of the F_1F_o-ATPase. This allele, *ATP1-111*, is dominant (Fig. 4 and data not shown), and requires functional Atp2p and Atp3p subunits of F_1-ATPase to exert its suppressing effects, but not the stator stalk subunits Atp4p and Atp5p (Fig. 6). In this report and previously (Kominsky et al., 2002) we have observed that suppression of *yme1*Δ ρ° slow growth by mutations in *ATP1* and *ATP3* is associated with an increase in F_1-ATPase activity. In addition, the slow growth of *atp3*Δ ρ° is rescued by a mutation in *ATP2* that increases F_1-ATPase activity (Smith and Thorsness, 2005). The same mutation increases *yme1*Δ ρ° growth and converts *atp3*Δ *yme1*Δ, which is essentially inviable, into a viable ρ° strain (Smith and Thorsness, unpublished observations). Thus, the analysis of four independent mutations in three different F_1-ATPase subunits lead us to conclude that mutations that increase F_1-ATPase activity allow or improve growth of petite negative strains, presumably by acting in consort with ANT to increase mitochondrial membrane potential (Giraud and Velours, 1997). These results are difficult to reconcile with the observation by Chen and Clark-Walker (1999) that a suppressing mutation in the ß-subunit of F_1-ATPase has decreased mitochondrial ATPase activity compared to the isogenic wild-type. Perhaps variations in strain backgrounds, growth conditions, or stability of mutant F_1-ATPases in isolated mitochondria could account for the different observations.

Another particularly revealing observation is that ρ° yeast bearing the suppressing alleles in *ATP1* and *ATP3* genes grow substantially faster than yeast bearing the wild-type alleles (Table 2), indicating that growth of ρ° yeast in glucose media is limited by F_1-ATPase activity, and in turn limited by the ability to generate an adequate mitochondrial membrane potential. The same limitation is not present in ρ^+ yeast strains grown on glucose as alleles of *ATP1* and *ATP3* that increase the electrogenic flux through ANT do not impact the rate of growth, presumably due to the effectiveness of the electron transport chain and F_1F_o-ATPase at establishing a sufficient potential across the mitochondrial inner membrane.

Pleiotropic consequences of mutations in *YME1* and genes encoding subunits of the F_1F_o-ATPase with respect to growth rate, culture yield, aging and spore viability

The suppressing alleles of *ATP1* and *ATP3* that support respiratory growth (e.g. *ATP1-111*, *ATP1-75*, and *ATP3-1*) have a relatively minor negative impact on growth (Tables 2 & 3). When combined with *yme1*Δ the negative respiratory growth effects of these alleles are greatly enhanced (Table 2). In contrast, the same mutations have no discernable impact during log-phase growth in fermentable media. *ATP3-5* seems to be an extreme example of this differential effect, growing on fermentable media with growth rates similar to the other *ATP1* and *ATP3* mutant strains, while being completely incapable of respiratory growth. One possible explanation for the differential effects of the suppressor mutations on growth in fermentable and nonfermentable media could be that these mutations do not uncouple the pumping of protons and ATP hydrolysis during fermentation in the way that they apparently uncouple H^+ translocation and ATP synthesis during respiration. Another possibility is that the suppressors do uncouple H^+ translocation and ATP hydrolysis during fermentation, but that the loss of membrane potential due to the uncoupling is compensated by an increase in higher F_1-ATPase activity and the corresponding increase in the electrogenic flux through ANT.

Surprisingly, these suppressing alleles increase the time cells remain viable in stationary phase when they are respiring on ethanol (Fig. 7A). The basis for the increased viability of the suppressing alleles of *ATP1* and *ATP3* in ρ^+ cells is unknown, but may be related to the coupling of proton pumping and ATP synthesis through the altered ATP synthase. Increased CLS resulting from increased efficiency of electron transport and decreased ROS production has been induced artificially by addition of mild respiration uncouplers (Barros et al., 2004). Partial uncoupling of proton transport and ATP synthesis resulting from *ATP1* and *ATP3* mutations may produce a similar increase in respiration efficiency. If the increase in CLS for strains bearing suppressor alleles is due to increased efficiency of respiration, the same suppressing alleles would, by some unknown mechanism, be responsible for a decrease in respiration efficiency in the presence of yme1Δ producing shorter CLS (Fig. 7B).

The rapid demise of wild-type ρ° and *cat5*Δ ρ° yeast while aging in culture (Fig. 8) is associated with an increase in ROS (Fig. 9) generated from mitochondria (Fig. 10). This observation is consistent with the presence of a strong source of ROS during programmed cell death induced in ρ° cells by starvation for essential amino acids, and which may be generated by the truncated respiratory chain (Eisler et al., 2004). In contrast, acetic acid induced programmed cell death is abolished in ρ° cells (Ludovico et al., 2002). However, there is no increase in ROS for the short-lived *ATP3-5* ρ^+ yeast

suggesting that in this case the loss of viability is due to an inability to generate ATP in conditions requiring oxidative phosphorylation. Similarly, the shorter CLS in aged cultures of ρ° strains bearing suppressing mutations in *ATP1* and *ATP3* compared to wild-type ρ° strains (Fig. 8) might be due to increased ATP hydrolysis in mitochondria, leading to a more rapid depletion of cellular energy supplies. Similar effects may explain the decreased viability of aged spores derived from diploids containing heterozygous and homozygous alleles of *ATP1* and *ATP4* (Table 4). These mutations could lead to spores with decreased ATP reserves in mitochondria and throughout the cell, thus decreasing the probability of generating ρ^+ and/or viable spores.

Spore viability depends upon events that occur during sporulation, the post-sporulation period, and germination. Of these, only the pre-meiotic stage of sporulation has a requirement for respiration (Miyakawa et al., 1984; Treinin and Simchen, 1993). Our results showing that respiration is affected negatively by mutations in *ATP1* and the deletion of *YME1* suggest that the increased loss of spore viability of strains containing *ATP1-111* and *yme1*Δ is due to effects that occurred at that sporulation stage even though sporulation was still possible. Numerous energy-requiring activities occur during sporulation (Puglisi and Zennaro, 1971; Tingle et al., 1974; Newlon and Hall, 1978), the post-sporulated period (Brengues et al., 2002), and germination on glucose-containing media (Rousseau and Halvorson, 1973; Choih et al., 1977; Seufert et al., 1990; Xu and West, 1992; Herman and Rine, 1997; Brengues et al., 2002; Kono et al., 2005). The spores produced may be deficient in energy or energy reserves derived from ATP synthesis generated at the premeiotic stage, thereby decreasing the probability of generating ρ^+ and/or viable spores. The far lower spore viability of cells containing these mutations in combination with *atp4*Δ, which effectively eliminates F_o, supports the conclusion that deficiencies in oxidative phosphorylation are responsible for the loss of spore viability.

Early studies are conflicted on the question of whether or not spores lacking functional mtDNA can germinate (Palleroni, 1961; Seigel and Miller, 1971; Rousseau and Halvorson, 1973; Kuenzi et al., 1974; Saverese, 1974; Tingle et al., 1974; Newlon and Hall, 1978; Hartig and Breitenbach, 1980; Hartig et al., 1981). Recently, strains defective for mitochondrial fission and/or fusion events have been shown to complete meiosis and sporulation, but mitochondrial compartments are not inherited equally among the four spores of a tetrad producing a loss of spore viability, and nucleoids are not segregated evenly, producing petite colonies lacking mtDNA (Gorsich and Shaw, 2004). We found that ρ° colonies are produced at high frequency by spores of diploids heterozygous for *atp4*Δ. *atp4*Δ-containing colonies from these crosses are ρ°, but, unexpectedly, in some cases the *ATP4* colonies are also ρ°. It is possible that the germinating spores were initially ρ^+ and rapidly lost their mtDNA, but a more likely explanation is that these spores germinated without mtDNA because even some wild-type spores were ρ°. The proportion of tetrads in which all four spores were ρ° increased when *ATP1-111* replaced *ATP1*. The diploid must be ρ^+ at least during initial stages of sporulation as respiration is required during the premeiotic stationary phase (Olempska-Beer, 1987; Treinin and Simchen, 1993) and for replication during sporulation (Tingle et al., 1974). A functionally significant decrease in ATP synthase in diploids that are heterozygous for *atp4*Δ during sporulation would account for the haplo-insufficiency with respect to the formation of viable spores. When coupled with the dominant effect of greater rates of ATP hydrolysis when F_1-ATPase contains subunits encoded by *ATP1-111*, a substantial energy deficit may be experienced during sporulation, explaining the inability of mtDNA to be replicated and segregated in the sporulation of these diploid yeast.

Implications of *ATP1* and *ATP3* suppressing mutations on the mechanism of F_1F_o-ATPase

Based on the crystal structure of bovine F_1-ATPase, it has been hypothesized (Abrahams et al, 1994) and subsequently confirmed (Duncan et al., 1995; Sabbert et al., 1996; Noji et al., 1997; Omote et al., 1999) that ATP hydrolysis drives rotation of subunit γ (=Atp3p) within $(\alpha\beta)_3$ (=(Atp1p/Atp2p)$_3$). The crystal structure suggested a model in which the end of the C-terminal helix of Atp3p rotated within a 'hydrophobic bearing' formed by loops provided by Atp1p and Atp2p (Abrahams et al., 1994). An alternative model proposed that the end of the C-terminal helix of Atp3p was fixed to the hydrophobic bearing on the nanosecond time scale, and Atp3p rotation pivoted around a partially unwound segment of the C-terminal helix that is just N-terminal to the segment bound to the hydrophobic bearing (Muller et al., 2004).

This study and others (Clark-Walker et al., 2000; Kominsky et al., 2002) suggest that the interaction between the hydrophobic bearing and the C-terminal helix of Atp3p plays a role in coupling ATP synthesis/hydrolysis with proton translocation. If the steps in proton translocation coupled to ATP synthesis are precisely the reverse of those in proton translocation coupled to ATP hydrolysis, the effects of mutations should be the same for both reactions according to the principle of microscopic reversibility. In the model in which rotation occurs around a partially unwound part of the C-terminal α-helix of Atp3p, proton translocation coupled to the ATP synthesis is different from that coupled to ATP hydrolysis because unwinding of Atp3p's asymmetric C-terminal helix would be different depending upon the direction of rotation. Consequently, mutation of residues directly or indirectly involved in the unwinding process

would have different effects on respiration, which is what is observed for the mutants in this study. By analogy with the bovine structure, *ATP3-5* has a mutation in the C-terminal tail of Atp3p of an amino acid that interacts directly with the hydrophobic bearing. *ATP3-1* has a mutation in the C-terminal tail at a position that interacts with the edge of the hydrophobic bearing close to the proposed site of unwinding of the C-terminal helix of Atp3p. The suppressor mutations in Atp1p, phenylalanine for valine at position 111 and isoleucine for asparagine at position 102, incorporate more bulky hydrophobic side chains into residues that interact with the α-helix that is N-terminal to the loop of Atp1p which forms part of the hydrophobic bearing (Abrahams et al., 1994). These changes may indirectly alter binding of the C-terminus of Atp3p to the hydrophobic bearing and thereby affect the rotation of Atp3p.

How do the mutations in *ATP1* and *ATP3* affect respiration and fermentation differently? One possibility is that they disrupt coupling of proton translocation and ATP synthesis. Several examples of uncoupling of F_1 and F_o have been reported. In *E. coli* uncoupling has been observed for mutations in subunits a (equivalent to Atp6p) and c (Atp9p) that are believed to participate in proton conduction (Mosher et al., 1985; Fraga et al., 1994; Valiyaveetil and Fillingame, 1998). When subunits γ (Atp3p) and c are cross-linked, ATPase activity increases owing to release of F_1 from the constraints of F_o (Schulenberg et al., 1999). A mutation in *E. coli* subunit b (Atp4p) uncouples F_o and F_1-ATPase activity, suggesting that the stator stalk may be also involved in coupling (Caviston et al., 1998). Cross-linking of bovine γ and ß subunits uncouples ATP synthesis and proton translocation in one direction but has no effect on ATP hydrolysis and proton translocation in the opposite direction (Gaballo et al., 1999). Partial uncoupling is also observed in *S. cerevisiae* through heterozygous null mutations in the Atp3p and Atp16p that form the rotor of F_1 (Xiao et al., 2000). Uncoupling via altered interactions between Atp1p and Atp3p could explain the growth characteristics observed. Uncoupling produced by altered interaction of the C-terminal helix of *ATP3-5* and the hydrophobic bearing may be sufficient to prevent respiratory growth. These altered interactions may be transmitted to the interface between Atp3p and Atp9p and thereby affecting coupling, but, more likely, they are transmitted to the proposed site of helix unwinding in the C-terminus of Atp3p. If uncoupling requires more protons to be translocated per ATP synthesized by the mutants than wild type, ATP synthase becomes less efficient, and growth rate would slow.

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